STUDIES ON ANTIBACTERIAL PRODUCTS FORMED BY MOLDS

I. Aspergillic Acid, a Product of a Strain of Aspergillus Flavus[1]

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Received for publication September 25, 1942

In 1940 one of us (White) reported that a strain of *Aspergillus flavus*, grown on certain liquid media, yielded filtrates that showed antibacterial activity against certain gram-negative as well as gram-positive bacteria. This was the second reported case of such behavior by a mold, the first being that of Fleming (1929), who found that a strain of *Penicillium notatum* formed a product that was inhibitory, but not markedly lethal, to certain gram-positive bacteria. Fleming's mold has since been the subject of much further investigation, culminating in the preparation of a highly potent product by McKee and Rake (1942) and in the recent report by Meyer *et al.* (1942) which describes the isolation of an extremely potent, crystalline inhibitory substance, to which the name "penicillin" is given. The present paper presents details of the isolation of an active crystalline substance from liquid cultures of our strain of *Aspergillus flavus*. Glister (1941) has also reported obtaining a potent antibacterial concentrate from an *Aspergillus*, but apparently not in pure crystalline form. Whether the active part is the same as our product is not apparent at this time. We propose to call our substance "aspergillic acid."

THE MEDIUM

Our organism is not fastidious but will grow on almost any medium rich in organic matter. Fair growths occur on the usual Czapek-Dox solution, which is essentially sucrose and sodium nitrate. Good growth occurs on a solution of malt extract, corn-steep liquor solution, ordinary infusion broth, and tryptone or peptone solution, with or without the addition of glucose. Growth is scant if the nitrogen source is solely glycine or urea. However, heavy growth may occur, as on malt extract, without development of any antibacterial activity in the filtrate. Indeed, the only media in which this activity has been noted up to now are, in decreasingly favorable order, tryptone,[2] peptone and corn-steep liquor. The patterns of behavior as to organisms affected and titers are similar in these three media, but the gummy products formed when steep liquor is sterilized, and the difficulty of filtration, led to its elimination. Tryptone seemed to give somewhat better titrates than did peptone, and was, therefore,

[1] This work was carried out with the aid of grants from the American Association for the Advancement of Science and from the Fluid Research Fund of the Rockefeller Foundation.

[2] This was the Difco product, which is understood to be a tryptic digest of casein. No other casein digest was tried.

used regularly in the experiments here reported. Since luxuriant growth may occur without production of antibacterial activity, it is plain that formation of active substance is not an essential part of the mold's activity, but the result of enzymatic action upon particular substances or balance of substances in the nutrient solution.

Stock cultures of the mold were grown on Czapek-Dox agar. The stock solution contained 2 per cent Difco tryptone and 0.5 per cent sodium chloride. Higher concentration of the nutrient substances did not increase the antibacterial titres. Attempts were made to increase the concentrations of active product by enriching the medium after the first inoculum had apparently reached maximal growth, in some cases removing the first mycelium and reinoculating. These efforts failed to produce much further growth or significantly higher titers. This may mean the presence of an autoinhibitory substance.

The addition of 0.5 per cent glucose caused somewhat more rapid growth, and in general gave slightly higher titers. However, in the isolation of the active crystalline material to be described the yield, which is extremely variable, was by far the best in one particular experiment in which no glucose was used and the growth of mycelium was noted as lighter than usual.

TITRATION OF FILTRATES

The nutrient solution is inoculated in Blake bottles lying on their sides, so that the depth of layer is about 1 or 1.5 cm. No relationship could be found between age of culture or heaviness of inoculum and titer. Usually a slant culture about two weeks old was used. Each 40 ml. solution was inoculated with two 4 mm. loop scrapings, the inoculation being made in such a way as to keep the spores on the surface. With larger volumes a suspension of spores was used. Growth is rapid. If the inoculum is evenly spread, mycelium will cover the surface in three days, and sporulation is generally evident in five or six days. Maximal titers are generally reached at this time. The reaction, which is acid during the first few days of growth, particularly if a sugar is present, is now alkaline, reaching a point between pH 8.0 and 9.0. The mycelium is filtered off and the filtrate is neutralized by stirring in concentrated HCl drop by drop with vigorous stirring. Fumes of ammonium chloride appear, and before neutrality is reached a small amount of a gummy precipitate is formed. This cannot be completely redissolved in dilute alkali. Repeated examination of these alkaline extracts as well as of extracts made with various solvents have shown no activity; hence the gum has been discarded. The filtrate, which has suffered an undetermined depletion by the mold growth, is enriched by the addition of 0.5 per cent tryptone and 0.5 per cent glucose, and is Seitz filtered. The freezing point depression at this point lies between 0.7 and 0.9°C.; thus the physical-chemical factors of reaction and osmotic pressure are favorable to the growth of all the organisms tested, and control experiments have shown that the amounts of nutrient added as enrichment are of themselves sufficient to give, with the inocula used, visible growth after over-night incubation of all the organisms used.

The Seitz filtrate is tubed in 1 ml. portions. Serial dilutions are made with a

diluent containing 0.5 per cent each of Difco tryptone, glucose and sodium chloride. To each tube to be inoculated is also added, in the case of the hemolytic streptococcus, a drop of 1:1 rabbit blood and normal saline mixture. Controls for growth in the diluent alone are run in each experiment. Growth of the hemolytic streptococcus in this medium produces a muddy suspension which is always checked by a smear. The inoculum is a 4 mm. loopful of an undiluted 18-hour culture. When the same amount of a 1:10000 dilution of culture was used, titers for all organisms were very little higher than with the heavy inoculum. Readings for growth were made after 18 hours incubation.

The filtrates have been tested against the following organisms: (a) *Pseudomonas aeruginosa*, (b) *Proteus*, (c) *Staphylococcus aureus*, (d) group A beta hemolytic streptococcus, strain 203, (e) *Streptococcus fecalis*, (f) *Escherichia coli*, (g) *Aerobacter aerogenes*. No inhibition was noted in the case of the first two organisms, even with the dilute inoculum. The results with the other organisms were variable. As a rule the undiluted Seitz filtrate inhibited them all. *S. aureus* has been inhibited as high as 1:40, as has the hemolytic streptococcus, but often only at 1:10; *S. fecalis* as high as 1:10, but generally lower; *E. coli* and *A. aerogenes* generally no higher than 1:5. Although subcultures after 18 hours incubation are frequently negative, such sterilization has been so variable both as to organisms and titers that a tabulation would not be justified.

At certain periods it has been noted that the titers fell off sharply, and there was little activity beyond the undiluted filtrate. Later cultures showed increased activity. This variation in titer as well as of the yield of active crystalline material to be described below raises the question whether the mold undergoes a series of spontaneous mutations. Since another mold examined during the same period has shown relatively constant behavior it seems improbable that unsuspected contaminants or unrecognized variations in technique are the explanations.[3]

ISOLATION OF CRYSTALLINE ASPERGILLIC ACID

When an active mold filtrate, unenriched, is treated at pH 4.0 with 2 per cent of an active charcoal, such as Merck's blood charcoal (now unavailable) or Norite, the filtrate after neutralization and enrichment with tryptone and glucose permits the growth of all the organisms employed. Adsorption of the active material also takes place, but not as completely, at neutrality. After standing several hours with frequent stirring the charcoal is filtered off and air-dried for a day. It is then exhausted with ether in a Soxhlet extractor. The extract is a red solution which, on spontaneous evaporation, leaves a red gum.

[3] Dr. G. W. Rake and Miss H. Jones, studying this mold at the Squibb Institute for Medical Research, have noted similar variations. They permit us to quote them as follows: "Careful study has shown that the parent culture of *A. flavus* used in the present studies was greatly subject to variation or mutation, particularly on certain media. Study of numerous variants has revealed that in some no production of aspergillic acid may occur, while others give maximal titres in 72 hours. Strains have been selected which give such maximal activity within a short time, and all present work is being carried out with these."

When the amount of aspergillic acid present is relatively larger the gum may, on standing in the cold, become suffused with a feathery mass of crystals. The mass is extracted at about 50°C. with 2 per cent sodium bicarbonate solution, using as little as is necessary to keep the mixture distinctly alkaline and stirring the melted gum vigorously. The gum is insoluble in the solution, which becomes yellow, and itself shows no activity whatever in extracts in various solvents. The solution is repeatedly filtered through a small wet paper until clear, and is brought to an acidity of about pH 4.5, or until no further precipitation occurs. Depending on the amount of material present, an opalescence or actual precipitation may occur, and in many cases the milky suspension forms crystals on stirring or standing in the cold. Excess acid must be avoided, for the precipitate is an amphoteric substance which also dissolves on the acid side.

The material can be purified by reprecipitation from alkaline solution and crystallization from dilute alcohol. In the latter procedure very little alcohol must be added, and a relatively large amount of water must be used to reprecipitate the product. The yields have been small and very variable, corresponding roughly to the titers of the filtrates. Batches of 3 liters have yielded from a mere opalescence, which did not crystallize, up to 200 mgm. This last yield was obtained only once, and generally no more than 15 or 20 mgm. could be recovered. The small yields precluded crystallization beyond the steps described. However, the material so obtained showed the following properties. The melting point has varied between 84° and 96°C., the latter being obtained in four preparations. On one occasion salting out after acid precipitation gave a small amount of a product melting at about 116°.[4] A sample melting at 96° showed no change on another crystallization from dilute alcohol. The crystals have a pale cream color and a characteristic, pleasant and rather "sweet" odor. A blank experiment, in which acidified tryptone was absorbed on charcoal and extracted as above, gave neither gum nor crystals. The appearance of these crystals under low power is shown in Plate I. They are insoluble in water but melt readily when warmed with it, and soluble in warm sodium bicarbonate solution; solution is best effected by adding to the dry acid about half its weight of sodium bicarbonate and a little water and stirring with gentle warming. Acid reprecipitates aspergillic acid unchanged, sometimes as an emulsion, which redissolves in acid. The substance is very readily soluble in alcohol, ether, acetone, glacial acetic acid, benzene, chloroform, carbon tetrachloride or pyridine, but insoluble in petroleum ether. Attempts to precipitate other solutions with petroleum ether gave only a sticky material which did not crystallize.

The amphoteric nature of aspergillic acid and the fact that its acid solution yields a bulky precipitate with phosphotungstic acid, indicates that it contains nitrogen. However, sodium fusion gave negative results, possibly because of

[4] Similar variations in melting point have been noted in highly purified products both in the Lederle Laboratories and in the Squibb Institute for Medical Research. These different products have shown the same composition on analysis, and have been constant in antibacterial activity. Although this question has not yet been settled, it is believed probable that we are dealing with a case of polymorphism.

the small amount available for the test. Phosphorus and sulfur are absent. Diązotizable nitrogen is absent, and no color is produced by a diazo solution, either in acid or alkaline solution. The Millon, xanthoproteic, Molisch, biuret and ninhydrine reactions are all negative. Ferric chloride gives a wine-red color in dilute alcoholic solution and a rusty precipitate may form if too little alcohol is present. Mercuric chloride forms no precipitate in alcoholic solution, but mercuric acetate forms a micro-crystalline precipitate which, washed free of excess mercury salt, reacts immediately with sodium hydroxide to give the yellow oxide. Silver nitrate forms a fine crystalline precipitate. The barium and calcium salts are soluble. There is no reaction with dinitrophenylhydrazine, indicating the absence of carbonyl groups, and there is no reduction of alkaline permanganate in the cold.

After the charcoal extraction with ether, exhaustion was also carried out with acetone, alcohol and dilute sodium carbonate. The acetone extract showed only slight antibacterial activity, probably representing a residuum not removed by ether, rather than another product. The other extracts showed no activity.

As the amounts of aspergillic acid that could be produced in this laboratory did not suffice for animal experimentation or even for sufficient purification to obtain a product suitable for analysis, the cooperation of the Lederle Laboratories, and later of the Squibb Institute for Medical Research, was enlisted. Both these laboratories have confirmed our isolation of aspergillic acid and demonstrated its antibacterial properties. Drs. Y. Subbarow and N. Bohonos at the Lederle Laboratories have used essentially our procedure, and have submitted the product to an elaborate purification involving several crystallizations, formation of the hydrochloride by saturating the ether solution with HCl gas, liberation of the free acid and molecular distillation. They kindly permit us to quote the following analytical results:

> Titration with alkali showed an equivalent weight of 222. Elevation of the boiling point of methanol gave molecular weights of 222 and 218. Elementary microanalysis: found C 64.38, H 9.15, N 12.76, 12.99 per cent. Calculated for $C_{12}H_{20}N_2O_2$ (molecular weight 224) C 64.29, H 8.88, N 12.5 per cent.

This material seemed identical with our product in every way except that it melted at a slightly lower point. We tested it for antibacterial activity according to the method used in table 1, and the results agreed almost identically with those found when we used material of our own preparation.

Quite independent preparations of aspergillic acid at the Squibb Institute by Menzel, Rake and Wintersteiner (1942) have also shown a molecular weight of 224 and an elementary analysis indicating the empirical formula $C_{12}H_{20}N_2O_2$. This product also appears identical with ours. The combination of composition and properties of this substance matches those of no known compound. At the time of writing nothing is known of its structure.

ANTIBACTERIAL ACTIVITY OF ASPERGILLIC ACID

The antibacterial activity of the crystalline aspergillic acid under two sets of conditions is shown in tables 1, 2, and 3. The tests were made with material

that was obtained in a yield of 22 mg. from 1500 ml. of culture medium and that had been twice crystallized from carbonate solution by precipitation with acid and once crystallized from dilute alcohol. The dilutions shown represent the amount of crystalline aspergillic acid converted to the sodium salt and diluted in the test medium.

Series I. Qualitative tests

The aspergillic acid was diluted with a solution containing 0.5 per cent each of tryptone, glucose and sodium chloride. This is the same diluent as was used in titrating the crude filtrate. In tests with the hemolytic streptococcus a drop of rabbit blood was added to every tube before inoculation. The inoculum in each case was a 4 mm. loopful of undiluted 18-hour culture to 1 ml. Readings were made after 18 hours of incubation and transfers were then made to optimal media. The results are shown in table 1. The first figure in each block of this table indicates whether there was inhibition or growth; the second figure shows

TABLE 1

The activity of aspergillic acid against heavy inocula

DILUTION	STAPH. AUREUS	BETA HEMOLYTIC STREPTOCOCCUS	STREP. FECALIS	E. COLI	AEROBACTER AEROGENES
1:1000	0 0	0 0	0 0	0 0	0 0
1:5000	0 0	0 0	0 #	0 #	0 0
1:10,000	0 #	0 0	0 #	0 #	0 #
1:20,000	0 #	0 #	0 #	sl #	0 #
1:40,000	0 #	0 #	#	#	#
1:80,000	#	#	#	#	#

Inoculum, a 4 mm. loopful of undiluted 18 hour culture per ml.
sl = slight growth as compared with next higher dilution.

the result of transfer. Thus, 0 0 means sterilization, 0 # means inhibition of growth without sterilization, and # means no apparent activity.

The striking feature of this table is that under the conditions of this test the effect of aspergillic acid does not vary greatly among the five pathogens subjected to its action. This is in contrast to the behavior of the crude filtrate with which a relative specificity was shown against *S. aureus* and the hemolytic streptococcus. This may mean that the extraction of the active materials absorbed on charcoal was not complete and that, therefore, materials other than aspergillic acid may not have been recovered. Alternatively, it may mean that there may have been present in the crude filtrate substances which partially inactivated the aspergillic acid as to its effect on some organisms, but not on all. Such a mechanism would parallel the behavior of para-aminobenzoic acid with the sulfonamides.

Series II. Quantitative tests

A. The action of aspergillic acid on bacteria in infusion broth. The direct antibacterial action of the drug was determined numerically by the tests shown in

table 2. The medium, beef-heart-infusion broth, pH 7.4, with or without aspergillic acid in dilutions of from 1:25,000 to 1:400,000, was tubed in 20 ml. amounts and inoculated with 0.2 ml. of saline dilutions of the test organisms. The number of colonies per ml. of the mixtures was determined by plating. In this

TABLE 2

The action of aspergillic acid on bacteria in infusion broth

ORGANISM	ASPERGILLIC ACID DILUTION	NUMBER OF COLONIES PER ML.				
		Start	2 hours	4 hours	8 hours	12 hours
Staph. aureus	1:50,000	36	0	0	0	0
	1:100,000	40	52	32	0	0
	1:200,000	24	14	<10	290	940
	1:400,000	22	40	220	1,000	6,540
	0	32	94	1,340	460,000	50,880,000
E. coli	1:25,000	102	0	0	0	0
	1:50,000	108	38	160	134,830	22,132,000
	0	110	635	20,990	31,300,000	181,260,000
A. aerogenes	1:50,000	210	0	0	0	0
	1:100,000	192	36	305	239,800	12,720,000
	0	186	425	7,125	19,080,000	223,235,000
Group A Strept. 203*	1:25,000	12	10	0	0	0
	1:50,000	22	12	44	100	<100
	1:100,000	8	46	340	22,000	612,000
	0	24	54	810	50,000	1,000,000
Pneumococcus Type I*	1:25,000	50	0	0	0	0
	1:50,000	114	256	816	24,000	760,000
	1:100,000	140	310	895	74,000	808,000
	1:200,000	112	272	1,200	126,000	980,000
	1:400,000	132	228	1,465	(78,000)	3,360,000
	0	150	246	1,500	148,000	6,400,000
g-*Strept. fecalis**	1:25,000	42	32	24	12	0
	1:50,000	24	132	342	930	311,600
	1:100,000	52	96	230	1,030	194,000
	1:200,000	44	96	280	1,830	420,000
	1:400,000	48	226	1,040	82,000	3,052,800
	0	40	280	1,490	158,000	7,250,000

* 0.25 cc. of defibrinated rabbit blood per 10 ml. of broth.

way the relative orders of growth could be determined. Light inocula were used in order to study the action of the drug under such conditions as compared with the heavy inocula employed in Series I, and in order to have a basis of comparison of the action of aspergillic acid with that of the sulfonamides.

It is seen from table 2 that an aspergillic acid dilution of 1:25,000 was directly bactericidal for 5 of the test organisms within 12 hours; 1:50,000 killed *S. aureus*

in 2 hours, but was only bacteriostatic for the other organisms. Dilutions of 1:100,000, 1:200,000, and 1:400,000 were consistently bacteriostatic as compared with the order of growth in the drug-free controls. Until enough material is available to test additional strains, conclusions cannot be drawn in regard to specific differences in the action of the drug on these organisms.

B. Comparison of the action of aspergillic acid and sulfanilamide. By a method previously used by Hill and Mann (1942) comparison was made of the action of 1:40,000 aspergillic acid and 1:10,000 sulfanilamide on *E. coli* in a synthetic glucose-asparagin medium (Bliss and Long, 1941). The results of such experiments are shown in table 3, and indicate that under the conditions of these experiments the direct killing action of aspergillic acid is greater than that of sulfanilamide.

TABLE 3

Comparative action of aspergillic acid and sulfanilamide on E. coli in a glucose-asparagin synthetic medium

DRUG	DRUG DILUTION	NUMBER OF COLONIES PER ML.				
		Start	2 hours	4 hours	6 hours	8 hours
Aspergillic acid	1:40,000	510	135	0	0	0
Sulfanilamide	1:10,000	510	605	1,445	3,600	7,500
Control	0	530	620	2,035	11,600	92,540

TOXICITY

Aspergillic acid was administered to mice *per os* both in solution as the sodium salt and in a suspension of the free acid in 10 per cent acacia solution. There was little difference in toxicity between the two forms. The tolerated dose was about 4 mgm. per 20-gram mouse, although a few mice died with this dose. With doses of 5 mgm. or more per 20-gram mouse, the animals went into convulsions in 5 to 10 minutes and died. Repeated doses of 4 mgm. per 20-gram mouse could be given at 6 hours intervals with survival and daily doses of this size were tolerated for at least a week.

Intraperitoneal injections of the sodium salt caused rapid onset of convulsions after doses of 3 mgm. per 20 grams and death within 10 minutes. Results with 2 mgm. per 20 grams were variable and 1 mgm. per 20 grams caused no reaction.

The rapid onset of symptoms, by whatever route the drug is administered, indicates that aspergillic acid is highly diffusible. This is in accord with its low molecular weight and extreme solubility in practically all solvents.

A mouse was given daily oral doses of 4 mgm. per 20 grams for one week, sacrificed and sections of all of its organs were examined microscopically by Dr. George Strong of the Department of Pathology. He reported no abnormality of any kind. There was a similar absence of pathologic change in a mouse that was killed by an intraperitoneal injection of 3 mgm. per 20 grams.

All of these results indicate that the toxicity for mice is largely confined to the nervous system and that with survival doses aspergillic acid is rapidly excreted or detoxified.

ANIMAL INFECTION

No therapeutic effect whatsoever was observed in mouse experiments in which the intraperitoneal inoculum was 0.5 ml. of a 1:500,000 broth dilution of cultures of either Group A hemolytic streptococcus 203 or Pneumococcus Type I, of which cultures of standardized virulence were obtained through the courtesy of Dr. Eleanor Bliss. Treatment by mouth consisted of initial doses of 2 or 3 mgm. per 20-gram mouse followed by repeated doses of 2 mgm. per 20-gram mouse every 4 or 5 hours thereafter during the survival period of the animals. The organisms were invariably recovered in pure culture from the heart blood of the treated animals and no evidence of any delay of death was observed. No effect was observed when treatment consisted of repeated intraperitoneal injection of 1 mgm. per 20-gram mouse, beginning at the time of inoculation. In smaller series no therapeutic action was observed in mice inoculated with *P. aeruginosa* or *S. aureus*. Four of six mice, however, survived intraperitonal inoculations of gonococci by the mucin technique (McLeod, 1941) although all 6 of the controls died within 48 hours. This small series is merely suggestive, and results of treating a much larger series of gonococcus infections will be reported later.

At the time of writing we have been informed by workers at the Squibb Institute for Medical Research that aspergillic acid has shown protective action against experimental gas gangrene infections. We are permitted to quote them as follows (Hamre, McKee and Rake, 1942).

"Although the toxicity of aspergillic acid is such that it is doubtful whether it will play an important role in systemic infections, it has been possible to demonstrate that the substance may play a role in the local therapy of gas gangrene."

SUMMARY

From cultures of a strain of *Aspergillus flavus* on tryptone solutions a crystalline substance, aspergillic acid, has been isolated. This substance shows antibacterial activity against certain gram-negative as well as gram-positive bacteria.

Aspergillic acid is an amphoteric substance of the empirical composition $C_{12}H_{20}N_2O_2$. It is of relatively high toxicity, and has shown no protective action against mouse infections with hemolytic streptococci or pneumococci. A short series which will be extended has shown protective action against infections with gonococci and mucin, and workers elsewhere have reported protective action in guinea pigs against gas gangrene.

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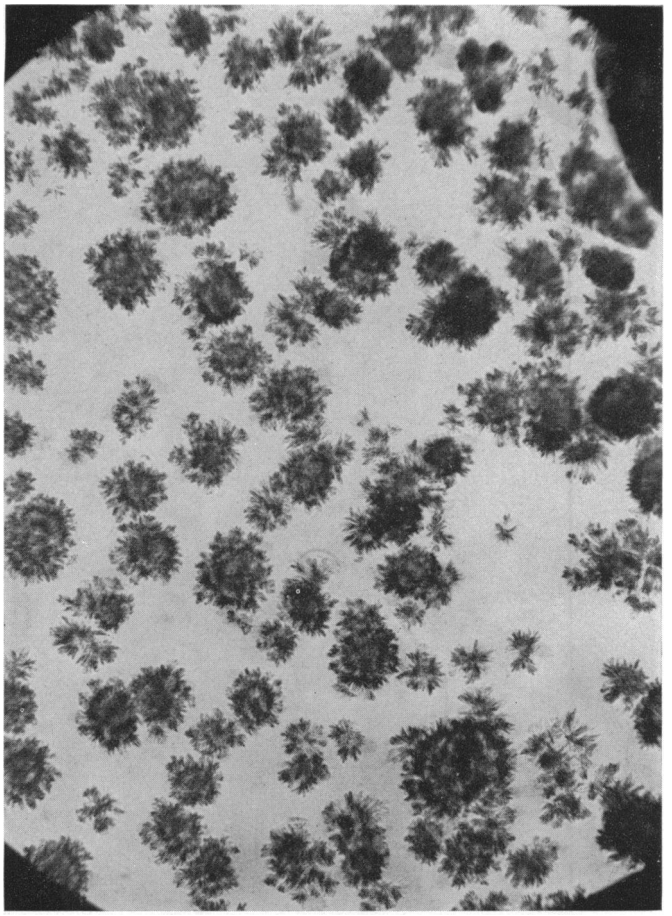
PLATE 1

ASPERGILLIC ACID, FROM ETHER SOLUTION (LOW POWER)



(Edwin C. White and Justina H. Hill: Studies on Antibacterial Products Formed by Molds)